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Components of normal serum block the focal segmental glomerulosclerosis factor activity in vitro

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Background. Sera from some patients with focal segmental glomerulosclerosis (FSGS) increase glomerular albumin permeability (P_{alb}) in vitro. The hypothesis that a component of normal serum can protect the glomerular permeability barrier was tested using sera from FSGS patients, normal individuals, and several mammalian and avian species.

Methods. In most experiments, isolated rat glomeruli were incubated in medium containing FSGS serum known to increase P_{alb} in vitro, normal serum, or both active FSGS and normal serum. In other experiments, fractions of normal serum and serum from other vertebrate species were incubated with active FSGS serum. P_{alb} was calculated from glomerular capillary expansion in response to an oncotic gradient. To enrich the blocking activity, normal pooled human plasma was subjected to various biochemical manipulations.

Results. Normal human serum prevented the increase in P_{alb} (active FSGS sera, 0.77 ± 0.12; active FSGS sera:normal serum, 1:1 mix, 0.06 ± 0.30, $P < 0.001$). Protection diminished as the concentration of normal serum was decreased. Specific fractions of human serum, including human albumin and immunoglobulin fractions, were not protective. Blocking activity was present in 80% ammonium sulfate precipitate and certain fractions from size-exclusion chromatography of normal pooled human plasma. Normal serum from each of the vertebrate species tested also prevented the increase in P_{alb}. Preincubation with normal serum was protective during subsequent incubation with FSGS serum, but normal serum was not protective after preincubation with FSGS serum.

Conclusions. We conclude that a factor or factors in normal serum block the permeability effect of active FSGS sera. This phenomenon may account for variability in proteinuria among patients with FSGS and may explain inconsistent proteinuria following injection of FSGS sera into experimental animals. Characterization of the protective substance(s) and the mechanism by which the increase in permeability is blocked may provide insight into the pathogenesis of FSGS.

Focal segmental glomerulosclerosis (FSGS) is characterized by nephrotic syndrome and a high incidence of progression to renal failure. It accounts for end-stage renal disease in many children and adults [1–8]. The observations that FSGS recurs in 30% of renal allografts, that proteinuria may begin within hours after transplantation, and that treatment with plasmapheresis can reduce proteinuria and stabilize renal function implicate a circulating factor as an etiologic agent in FSGS [9–11]. We have shown that sera from some patients with FSGS increase albumin permeability (P_{alb}) of isolated glomeruli in vitro, have isolated and partially purified this factor (FSGS factor), and have shown that the FSGS factor causes proteinuria in rats [12, 13]. Permeability activity has been confirmed by others [14, 15] and may predict recurrence [16, 17].

Our initial observations of increased P_{alb} caused by FSGS factor led us to hypothesize that proteinuria might be related to a deficiency of a protective substance in addition to the presence of an injurious one. The experiments described here address that hypothesis and have been carried out during the past 10 years concurrently with isolation of the FSGS factor.

METHODS

Determination of albumin permeability

Normal male Sprague-Dawley rats (120 to 150 g) maintained on rat chow and water ad libitum were used for all experiments. Kidneys were removed, and glomeruli were isolated in an isolation/incubation medium containing 4% bovine serum albumin (BSA) as an oncotic agent using standard sieving techniques. In most experiments, glomeruli were then incubated with FSGS serum with or without normal serum or fractions for 10 minutes at 37°C. In some studies, glomeruli were preincubated at 4°C for 10 minutes with normal serum or FSGS serum. An oncotic gradient across the glomerular capillary was produced by replacing the incubation medium with medium containing 1% BSA. Changes in glomerular volume consequent to this change of medium reflected expansion of glomerular capillaries and were recorded using videomicroscopy. Glomerular volume was calculated from the average diameter of the video image.

Key words: proteinuria, glomerular albumin permeability, nephrotic syndrome, filtration barrier, sclerosis, renal disease progression.

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Relative volume change (ΔV) was calculated as $\Delta V = (V_{Final} - V_{Initial})/V_{Initial} \times 100\%$. As previously described, the ratio of $\Delta V_{Experimental}/\Delta V_{Control}$ was used to calculate the albumin reflection coefficient (σ_{alb}) and convectional permeability (P_{alb}) where $P_{alb} = (1 - \sigma_{alb})$ [18].

Determination of blocking activity

To determine whether normal serum affects the increase in P_{alb} caused by the FSGS factor, studies were carried out using sera from FSGS patients, with active plasmapheresis fluid or fractions thereof; in each case, the substance tested had $P_{alb} > 0.5$. Normal serum was obtained from volunteers without known medical disease and was used individually or after pooling with two or more specimens. Additional studies were performed using serum of other species or fractions of human serum. In initial studies, glomeruli were incubated with serum of individual FSGS patients, with serum from normal volunteers, or with a mixture of normal human serum and serum from FSGS patients. The amount of each test substance added was 2% vol/vol. Details of the protocols are described with results of individual experiments.

Chemicals and supplies

Lyophilized powder of pooled serum from normal sheep, chicken, calf, mouse, cat, pig, duck, goat, or horse (Sigma Chemical Company, St. Louis, MO, USA) were reconstituted with sterile water. Fresh human and rat sera were prepared from whole blood. Purified human albumin and immunoglobulin fractions (Sigma Chemical Co.) were also tested. Additionally, larger amounts of normal human plasma were obtained from healthy volunteers or from a local blood bank (The Blood Center of Southeastern Wisconsin, Milwaukee, WI, USA). BSA (35% solution), high-pressure liquid chromatography (HPLC) grade solvents, analytical grade chemicals for buffers, and other reagents (Sigma), and Spectra-pore dialysis tubing (Spectrum Medical Industries, Inc., Laguna Hills, CA, USA) were used.

Enrichment of blocking activity

Normal pooled plasma was treated to remove cryoprecipitate, lipoproteins and chylomicrons, and protein fractions that are known to be rich in immunoglobulins and albumin using the same chemical manipulations as described earlier for enrichment of FSGS activity [10]. These steps are outlined briefly as follows: Plasma was frozen and allowed to thaw at 4°C; cryoprecipitate was discarded. Protein-bound lipids were removed by the addition of 10% dextran sulfate and 1 mol/L calcium chloride. The resulting precipitate of lipoproteins and chylomicrons was removed by centrifugation at 4°C and was discarded. The remaining plasma components were subjected to sequential ammonium sulfate precipitation at 50, 70, and 80% saturation. The supernatant at 70% ammonium sulfate saturation and the precipitate at 80% ammonium sulfate saturation were tested for blocking activity. Some of the 80% precipitate of normal plasma was subjected to size exclusion chromatography using Sephacryl S-300 matrix. Fractions of 1.5 mL size were collected and tested for blocking activity upon addition to the 80% precipitate of FSGS serum and the size exclusion (Sephacryl S-300) fraction of FSGS serum.

Electrophoresis

The 80% precipitates of active FSGS plasma and of normal human plasma were prepared as described earlier [10]. Aliquots of these two preparations containing equal amounts of protein and of a solution containing a 1:1 mix of each were subjected to electrophoresis under nondenaturing, nonreducing conditions (native conditions) using 4 to 20% gradient gels in Tris-glycine buffer.

Statistical analysis

Albumin permeability values are expressed as mean ± SD of the mean as indicated. *N* represents the total number of glomeruli for P_{alb} studies. Values among various groups were compared using Student's *t*-test or one-way analysis of variance (ANOVA), and significance was defined as $P < 0.05$.

RESULTS

Glomeruli were incubated with 2% vol/vol of normal human serum, with active sera from one of four patients with FSGS in native kidneys or recurrence in renal allograft or a 1:1 mixture of individual FSGS and normal sera. Incubation with normal serum alone (0.2 ± 0.1) did not alter P_{alb} values compared with medium without serum (0 ± 0.08). Incubation with each FSGS serum significantly increased P_{alb}. In each case, the increase in P_{alb} was prevented by the inclusion of normal serum. The characteristics of the patients studied and the results of incubation with their sera and with FSGS and normal serum are shown in Table 1. The effect of incubation with combined normal serum and negative FSGS patient serum, defined by failure to increase P_{alb} to more than 0.5, was also studied. In these experiments, P_{alb} was not altered by normal, negative FSGS, or combined sera (data not shown).

To determine whether the blocking effect of normal serum was species specific, serum from several vertebrate species was tested. In these experiments, serum from various species and FSGS serum were each added at concentrations of 2% vol/vol. Serum from all species tested prevented the increase in P_{alb} caused by FSGS serum. These results are shown in Table 2.

To determine the concentration of normal human serum required to prevent the increase in P_{alb} caused by FSGS serum, we added normal serum in concentrations

Table 1. Effect of normal sera on the increase in glomerular albumin permeability (P_{alb}) caused by focal segmental glomerulosclerosis (FSGS) sera

Patient number	Primary/recurrent	Gender	Age *years*	P_{alb} FSGS	P_{alb} FSGS + normal
1a, sf9091	Recurrent	M	30	0.70 ± 0.05 (5)	0.03 ± 0.05 (5)[a]
1b, sf171				0.85 ± 0.14 (5)	0.24 ± 0.20 (5)[a]
2, sf33	Primary	F	42	0.80 ± 0.06 (5)	0.03 ± 0.31 (5)[a]
3, sf95	Recurrent	M	48	0.64 ± 0.26 (5)	0.00 ± 0.47 (5)[a]
4, sf215	Primary	F	16	0.86 ± 0.08 (5)	0.01 ± 0.50 (5)[a]
Average P_{alb}				0.77 ± 0.12 (25)	0.06 ± 0.30 (25)[a]

Five active serum samples from four patients were tested for their capacity to increase P_{alb} of isolated glomeruli. Two patients had primary FSGS, two recurrent FSGS after renal transplantation as indicated. Values for P_{alb} are expressed as mean ± standard deviation with (N) equal to the number of glomeruli studied.

[a] Different from FSGS serum alone, $P < 0.01$

Table 2. Effect of serum from vertebrate species on the FSGS serum mediated increase in P_{alb}

Source of serum	*N*	Albumin permeability (P_{alb})
Sera from fresh blood		
None (FSGS patient only)	26	0.62 ± 0.18
Pooled human	6	0.00 ± 0.13[a]
Rat	15	0.05 ± 0.21[a]
Reconstituted sera		
Mouse	4	0.04 ± 0.20[a]
Cat	5	0.17 ± 0.28[b]
Goat	5	0.16 ± 0.04[a]
Sheep	6	0.01 ± 0.27[a]
Pig	5	0.36 ± 0.30[b]
Cow	6	0.06 ± 0.19[a]
Horse	6	0.33 ± 0.23[b]
Chicken	6	0.33 ± 0.17[a]
Duck	5	0.05 ± 0.07[a]

Isolated rat glomeruli were incubated in medium containing active FSGS serum with or without a 1:1 mix of serum from vertebrate species and P_{alb} was calculated. Serum from each species tested prevented the increase in P_{alb} caused by FSGS serum. Values for P_{alb} are expressed as mean ± standard deviation with N = number of glomeruli.

[a] $P < 0.001$, [b] $P < 0.02$, vs. FSGS serum alone

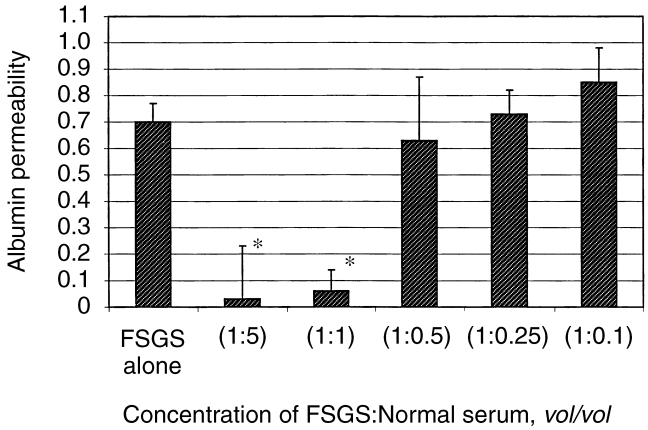

Fig. 1. Dose response of the blocking effect of normal serum on focal segmental glomerulosclerosis (FSGS) serum-mediated increase in glomerular albumin permeability (P_{alb}). Relative concentration of normal to FSGS serum of at least 1:1 is required to prevent increased P_{alb}. Values for P_{alb} are expressed as mean ± SD. The asterisk indicates different from glomeruli incubated with FSGS serum alone at $P < 0.001$.

of 10, 2, 1, 0.5, and 0.1% vol/vol to medium containing 2% FSGS serum. Inclusion of 10 or 2% normal serum, corresponding to relative concentrations of normal to FSGS serum of 5:1 and 1:1, respectively, completely prevented the increase in P_{alb}, while lower concentrations (relative concentrations of 0.5:1, 0.25:1, and 0.1:1) were not protective. These results are illustrated in Figure 1.

Studies were performed to determine whether the simultaneous presence of normal and FSGS sera is necessary to prevent the increase in P_{alb}. In these studies, glomeruli were preincubated with normal human serum for 10 minutes at 4°C followed by washing and subsequent incubation with FSGS serum from patients with recurrent disease for 10 minutes at 37°C. In another group, glomeruli were preincubated with the same FSGS serum for 10 minutes at 4°C followed by washing and subsequent incubation with normal serum for 10 minutes at 37°C. Additional glomeruli were also incubated either with normal serum or with FSGS serum throughout and served as negative and positive controls, respectively. Preincubation with normal serum protected glomeruli from increased P_{alb} caused by FSGS serum in subsequent incubation. In contrast, incubation of glomeruli with normal serum after preincubation with FSGS serum did not protect the glomeruli from the increase in P_{alb} caused by FSGS serum. Results of a typical experiment are shown in Figure 2.

To enrich the blocking activity, normal pooled human plasma was subjected to various biochemical manipulations, as outlined previously [12] and in the **Methods** section. Plasma retained its blocking activity after removal of cryoprecipitate, lipoproteins, and proteins precipitated at 50 and 70% of ammonium sulfate saturation. Blocking activity was present in both the supernatant at 70% saturation and in the 80% precipitate. In contrast, blocking activity was not found in commercially prepared albumin or in immunoglobulin fractions. The results of experiments using plasma fractions are displayed in Table 3.

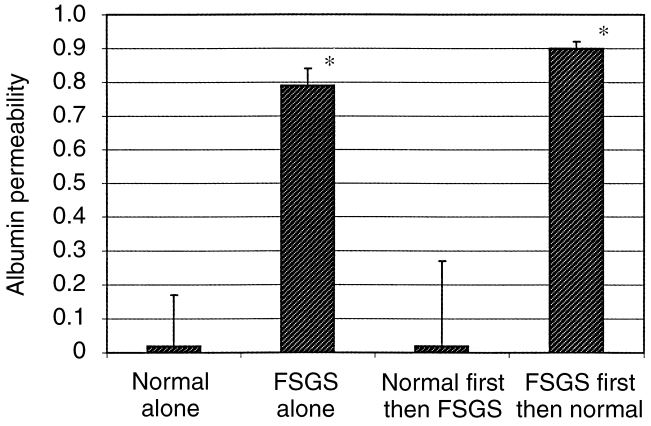

Fig. 2. Effect of sequential incubation with normal and FSGS serum on P_{alb}. Preincubation of isolated rat glomeruli with normal serum is required to protect them from the increase in P_{alb} caused by FSGS serum. Values for P_{alb} are expressed as mean ± SD. The asterisk indicates different from glomeruli incubated with normal serum alone or preincubated with normal serum prior to incubation with FSGS at $P < 0.001$.

Table 3. Effect of various fractions of normal plasma on FSGS activity

Fractions of normal plasma tested	Albumin permeability (P_{alb})	
	FSGS alone	FSGS + normal fraction
Human albumin	0.76 ± 0.05 (5), sf54	0.80 ± 0.16 (5)
Immunoglobulin	0.76 ± 0.05 (5), sf54	0.73 ± 0.15 (5)
80% precipitate	0.67 ± 0.07 (5), sf106	0.10 ± 0.23[a] (5)

Isolated glomeruli were incubated with active FSGS sera (sf54 and 106) alone (2% vol/vol) or with purified human albumin fraction (5% vol/vol), immunoglobulin fraction (5% vol/vol) and with 80% precipitate of normal human plasma (2% vol/vol). FSGS sera mediated increase in P_{alb} was blocked only by 80% ppt of normal pooled plasma. Values depicted for P_{alb} are mean ± standard deviation with (N) = number of glomeruli.

[a] $P < 0.004$, significant differences from P_{alb} with FSGS serum alone

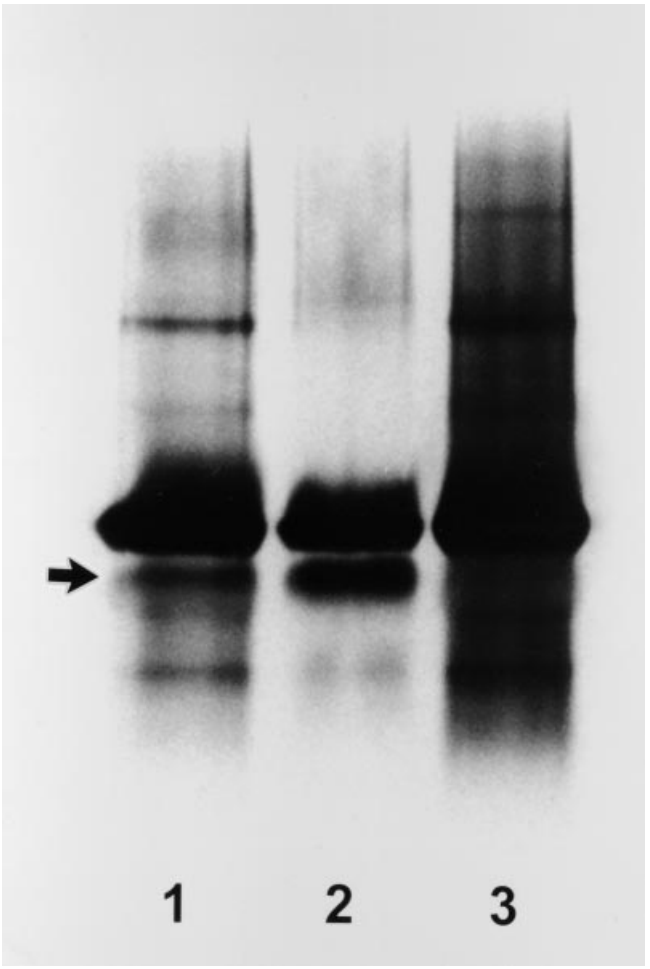

Fig. 3. Electrophoretic pattern of proteins in the enriched fractions of normal, FSGS, and a 1:1 mix of normal with FSGS plasma. Gel electrophoresis was performed under nondenaturing and nonreducing conditions, and proteins were stained using silver staining. Lane 1, 80% precipitate of normal plasma. Lane 2, 80% precipitate of FSGS plasma. Lane 3, a 1:1 mix of 80% precipitate of FSGS and normal plasma.

The 80% precipitate of normal human plasma, 80% precipitate of plasma from a patient with recurrent FSGS, or a 1:1 mixture of the two were studied for their protein profile by gel electrophoresis under nondenaturing, nonreducing conditions. Equal amounts of protein (100 ng) were loaded in each lane. As shown in Figure 3 and marked by an arrow, a prominent band is seen in the lane 2 (FSGS 80% precipitate). This band is less prominent in lane 1 (normal 80% precipitate) and is not evident in the lane 3 (1:1 mixture of FSGS 80% and normal 80% precipitate).

Active 80% precipitate from a patient with recurrent FSGS and an aliquot of 80% precipitate of normal plasma with blocking activity was dissolved in phosphate-buffered saline and passed through a Sephacryl S-300 size-exclusion column chromatography. Fractions of 1.5 mL were collected. All fractions of FSGS were tested for P_{alb} activity, and fractions 20 and 21 showed positive activity (0.53 ± 0.21, $N = 8$). All fractions of normal 80% precipitate were tested for blocking activity by incubating glomeruli with a 1:1 mix of each normal fraction and an active FSGS fraction 20. Only normal fraction 20 blocked the increase in P_{alb} caused by fraction 20 of FSGS (active fraction, 0.53 ± 0.21, $N = 8$; active FSGS + normal fraction, 1:1 mix, 0.18 ± 0.08, $N = 8$; $P < 0.001$). Normal fraction 20 alone had no effect on P_{alb} (0.19 ± 0.10, $N = 8$). Thus, fractionation of normal plasma revealed that the blocking activity was retained in the same fractions that carried the FSGS factor.

DISCUSSION

In the current studies, we confirmed our previous observations that sera from selected patients with FSGS either in native kidneys or after transplantation have the capacity to increase glomerular P_{alb} in vitro. We also

document that the increase in glomerular P_{alb} by FSGS sera is prevented by one or more components of normal serum. Protection is conferred by both individual human sera and pooled serum. In addition, plasma of each of the vertebrate species tested also prevented the FSGS-induced increase in P_{alb}.

The blocking effect depends on the concentration of serum used and is maximal when a concentration equal to or in excess of the concentration of FSGS serum is used. Thus, under our standard conditions of 2% FSGS serum, 2% or higher concentrations of normal serum were required. We found that the protective factor or factors need not be present in solution simultaneously with the FSGS factor to exert the protective effect. Specifically, preincubation with normal serum followed by washing and subsequent incubation with FSGS serum provided complete protection, while preincubation with FSGS serum followed by washing and incubation with normal serum still resulted in an increase in P_{alb}. We believe that competition between the FSGS factor and normal components for binding sites on glomerular cells is the most likely explanation for our findings. However, other mechanisms, including binding of a component of normal serum to the FSGS factor or enzymatic degradation of the FSGS factor by a component of normal plasma, cannot be excluded.

Protection was present both in intact serum or plasma and in specific plasma fractions. Blocking activity was retained during ammonium sulfate fractionation that removes the majority of albumin and immunoglobulins and was not present in commercially available purified human albumin or immunoglobulin fractions. The protective component of normal serum was present in the size-exclusion chromatography fraction analogous to that which contained the FSGS factor. These observations support the notion that the substance responsible for blocking permeability activity shares some biochemical characteristics with the FSGS factor itself.

The assay for glomerular albumin permeability has been developed and standardized in our laboratory during the past 12 years. Isolated glomeruli are observed as isolation medium is replaced by medium of lower oncotic concentration. Under constant experimental conditions, the resulting expansion of capillaries and increase in glomerular size are proportional to the reflection coefficient of albumin (σ_{alb}). For convenience, we have calculated convectional albumin permeability, P_{alb}, as $(1 - \sigma_{alb})$. Alternative causes for diminished capillary distention, including altered exchangeable volume caused by cell swelling and diminished capillary compliance, have been excluded using solutions of high molecular weight solutes such as dextran. Capillary responses to these gradients assure that alterations in volumetric responses to an albumin gradient are due to a change in permeability rather than other changes. The calculations and rationale for the calculation of P_{alb} have been detailed previously [18].

Our P_{alb} assay demonstrates the effects of several experimental interventions on the glomerular permeability barrier. Findings include documentation that (*1*) the C5b-9 membrane attack complex is essential to the immediate permeability increase induced by anti-Fx1A [19]; (*2*) antibodies to β_1 integrin increase permeability independent of complement [20]; (*3*) superoxide [21] or hydroxyl ions [22] increase permeability; (*4*) tumor necrosis factor-α [23] or transforming growth factor-β1 [24] increase permeability through superoxide; and (*5*) charge neutralization by protamine [18], prolonged incubation with platelet-activating factor [25], or enzymatic degradation of basement membrane by matrix metalloproteinase-3 [26] each increase permeability. In addition, we have used the assay to permit us to follow the permeability activity of FSGS sera through a series of biochemical steps to achieve partial purification of the FSGS factor [12].

We have previously shown that sera of about 30% of patients with FSGS increase P_{alb} to a value of greater than 0.5. Activity is enriched in fractions of plasma, specifically in 70% ammonium sulfate supernatant and precipitate at 80% ammonium sulfate saturation. An injection of 70% supernatant results in transient proteinuria in rats [12]. These findings are consistent with the reports of proteinuria after injection of patients' plasma [27] and certain plasma fractions eluted from protein-A column [13]. Seventy percent supernatant also inhibits induction of nitric oxide synthase (iNOS) in cultured rat mesangial cells [28] and thus confirms the presence of a substance that directly affects the function of glomerular cells.

The current findings that normal serum blocks the effect of FSGS serum on P_{alb} and that this effect is abolished if the ratio of FSGS to normal serum is highly supportive of the notion that a relatively large quantity of FSGS factor is required to overcome protective capability of plasma. The inconsistent results of injection of sera from FSGS patients may reflect the use of insufficient amounts of FSGS factor to overcome this protection. The mechanism for protection is not clear from the current experiments. We propose that the normal serum component and FSGS factor interact independently with glomerular cells. This interpretation is supported by our findings that sequential incubation with normal and FSGS sera results in maintenance of the filtration barrier, while initial exposure to FSGS serum followed by normal serum results in increased P_{alb}. Alternatively, it is possible that normal serum contains a component that binds to or enzymatically degrades the FSGS factor. The disappearance of a band on the gel electrophoresis of nondenatured proteins from comparable fractions of FSGS and normal plasma supports this hypothesis.

The fact that the physiologic response of glomeruli to

FSGS factor is prevented by the blocking factor in normal serum is not unique in that we have shown that cyclosporine A [29], indomethacin (abstract; McCarthy et al, *J Am Soc Nephrol* 7:2465, 1996), or derivatives of Tripterigium wilfordii [30] each prevent the increase in P_{alb} caused by the FSGS factor. Since the protection occurs in the absence of perfusion, we postulate that these agents prevent the increase in P_{alb} by acting on the cells of the filtration barrier. We further propose that a component(s) of normal plasma also prevents changes in glomerular permeability through action on glomerular cells.

In summary, the final effect of the FSGS factor on glomerular filtration may reflect the net influence of the FSGS factor and of protective substances found in plasma. The identification and characterization of the FSGS factor and of protective factors will be essential to understand fully the events leading to proteinuria and sclerosis in FSGS and other progressive renal diseases.

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